

Empirical Methods for Identifying Specific Peptide-protein Interactions for Smart Reagent Development

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Dimitra N. Stratis-Cullum, and Paul M. Pellegrino**

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Empirical methods for identifying specific peptide-protein interactions for smart reagent development

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ABSTRACT

The current state of the art in the development of antibody alternatives is fraught with difficulties including mass production, robustness, and overall cost of production. The isolation of synthetic alternatives using peptide libraries offers great potential for recognition elements that are more stable and have improved binding affinity and target specificity. Although recent advances in rapid and automated discovery and synthetic library engineering continue to show promise for this emerging science, there remains a critical need for an improved fundamental understanding of the mechanisms of recognition. To better understand the fundamental mechanisms of binding, it is critical to be able to accurately assess binding between peptide reagents and protein targets. The development of empirical methods to analyze peptide-protein interactions is often overlooked, since it is often assumed that peptides can easily substitute for antibodies in antibody-derived immunoassays. The physico-chemical difference between peptides and antibodies represents a major challenge for developing peptides in standard immunoassays as capture or detection reagents. Analysis of peptide presents a unique challenge since the peptide has to be soluble, must be capable of target recognition, and capable of ELISA plate or SPR chip binding. Incorporating a plate-binding, hydrophilic peptide fusion (PS-tag) improves both the solubility and plate binding capability in a direct peptide ELISA format. Secondly, a solution based methods, affinity capillary electrophoresis (ACE) method is presented as a solution-based, affinity determination method that can be used for determining both the association constants and binding kinetics.

Keywords: peptide ELISA, PS-tag, peptide affinity reagent, protective antigen, peptide capillary electrophoresis, immunoassay,

1. INTRODUCTION

Peptide affinity reagents have emerged as antibody alternatives using peptide display techniques such as bacterial display^{1, 2, 3, 4, 5}, yeast display^{6, 7}, phage display^{4, 5, 8, 9, 10}, and synthetic libraries¹¹, to isolate new reagents for therapeutics and diagnostics. In bacterial display technologies, peptides can be isolated in as little as 2-4 days, which would enable rapid isolation for reagents against new or emerging bio-threats. Peptides can be mass produced synthetically in large quantities and have greater thermal stability than most current antibodies (unpublished results) making them an attractive alternative to antibodies. Peptide reagents have been shown to exhibit comparable results to antibodies and other detection assays^{12, 13}, especially as an alternative diagnostic tool and low cost fieldable test¹⁴.

One of the challenges to using peptides as a diagnostic tool is to integrate peptides into many of the current immunoassays. There are few instances in the literature detailing the use of peptides as capture agents in enzyme-linked immunosorbent assays (ELISAs), and poor peptide performance in direct ELISAs is often described for peptides less than 20 residues in length. At less than 20 residues, the peptides have a limited number of sidechains to simultaneously recognize the target of interest and bind to the ELISA plate¹⁵⁻¹⁷, in addition to steric restrictions at the plate surface. Examples of direct peptide ELISA methods improved adsorption and binding capability include direct covalent attachment of peptides to a modified plate surface^{18, 19} or using a biotinylated peptide with a streptavidin or neutravidin plate²⁰. Indirect ELISA and competition ELISA²¹⁻²³ are often employed for ELISA analysis of peptides since direct methods had shown limited success. Indirect ELISAs require additional assay steps: the use of sandwich formats, enzyme labeled secondary antibodies, or protein-peptide fusions, such as BSA-peptides conjugates²⁴.

Recently, we have shown that the addition of the PS-tag, isolated from the Flitrx® peptide display library²⁵, increased the adsorption of peptides to ELISA plate surface (polystyrene) and improved the measured ELISA signal in a direct peptide capture format compared to native peptide and biotinylated peptide²⁶. Currently, we are developing affinity capillary electrophoresis (ACE) methods with bacterial display peptides for a solution based method to

supplement surface bound assays such as ELISA and surface plasmon resonance (SPR) to further better understand peptide-target binding interactions in the absence of steric restriction due to surface interaction.

Affinity capillary electrophoresis (ACE) was first reported in 1992 to study receptor-ligand interactions and has expanded to include the study of protein-protein, peptide-protein, and peptide-peptide interactions²⁷. ACE is emerging as a solution based immunoassay alternative to ELISA and SPR, since both ELISA and SPR are surface bound techniques that limit the conformation of biomolecules²⁸ with peptides and proteins provides a solution-based, immunoassay alternative to ELISAs. ACE is a high speed method, high sensitivity, and easily automated method²⁹ to determine if an interaction is occurring between a target protein and binding moiety, typically antibodies, small molecules, or peptides. An interaction is often measured by a change in the electrophoretic mobility in the bound and unbound state and can be measured using UV detection or laser-induced fluorescence (LIF). LIF provides much greater sensitivity, including reports of measurements with as few as six molecules, whereas UV detection is limited to 0.1 to 1 ng of proteins³⁰ with UV detectors sensitive to about 10^{-6} M³¹. ACE by UV detection is label-free, relying on the native absorption of biomolecules, whereas LIF requires covalently linking a dye-label to one of the reagents.

In capillary electrophoresis, the electrophoretic mobility is dependent on the size and charge of the molecule. For peptide electrophoretic mobility, the Offord Model gave the best correlation in a 58 peptide data set, where the effective electrophoretic mobility μ_{eff} is related to the valence of the molecule and the molar mass by the function³²:

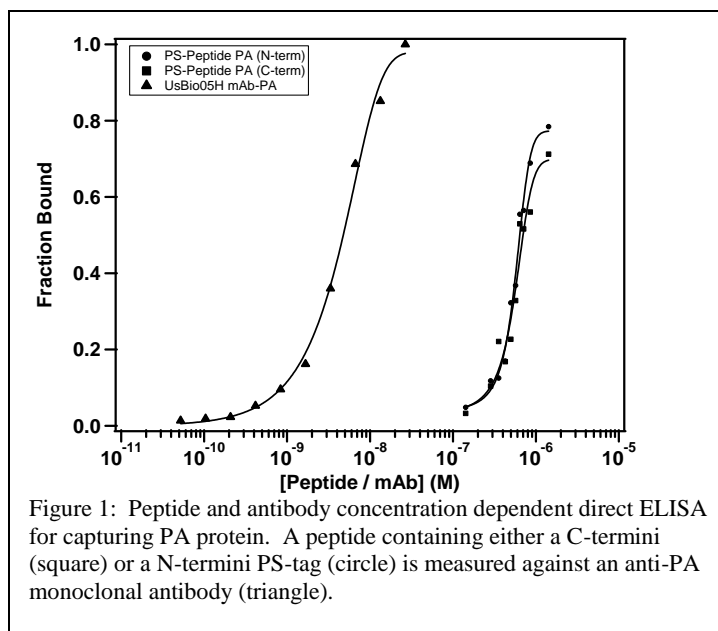
$$\mu_{eff} = q / M^{2/3}$$

Herein, two methods are presented to analyze the binding affinity of peptide reagents isolated from a bacterial display library, either direct peptide ELISA or peptide ACE. Both techniques were able to detect *B. anthracis* protective antigen protein (PA). For the direct peptide ELISA, the PS-tag peptide is compared directly with a PA antibody and shows comparable binding (Fig. 1). The utility of this PS-tag fusion peptide is further expanded to show that there is strong plate specificity for the PS-tag when exposed to typical ELISA proteins, as well as other *B. anthracis* proteins. The development of the ACE method focuses on the mobility differences between the use of a dye-labeled protein or dye-labeled peptide for LIF detection. Since the peptide mass and charge is expected to change most significantly upon binding, the peptide is hypothesized to be the best moiety to track during LIF ACE.

2. METHODS

2.1 PS-tag ELISA

2.1.1 Horseradish peroxidase labeling of protein targets: ELISA detection was completed using direct horseradish peroxidase labeling of all *B. anthracis* proteins presented using EZ-Link Plus Activated Peroxidase and Kit (Thermo Scientific) according to the manufacturer's instructions. The *B. anthracis* proteins, protective antigen (PA), edema factor (EF), and lethal factor (LF) were purchased from List Biological Laboratories, Inc. and required HRP labeling prior to analysis. HRP-conjugated streptavidin (strep-HRP) and NeutrAvidin protein, horseradish peroxidase conjugated (neutr-HRP) were purchased directly from Pierce Protein (Thermo Scientific) along with an anti-mouse IgG-HRP from US Biological for the HRP pre-labeled "cross-reacting protein" set.



ng PA detected per binding site	
mAb	Peptide
10.0	15.7
8.5	13.8
6.9	11.3
3.6	11.1
1.6	7.4
1.0	6.5
0.5	3.4
0.2	2.5
0.2	2.4
0.1	1.0

Table 1: The total amount of PA detected using mAb (2 equal binding sites) or peptide (1 site) as a capture agent in the presented direct ELISA format.

2.1.2 PS-tag direct peptide ELISA and anti-PA ELISA: ELISA analysis for the PS-tag peptide and the anti-*Bacillus anthracis*, Protective Antigen (mAb) were completed using a Maxisorp (Nalge Nunc; Rochester, NY) 96-well plate by initially dissolving each peptide at 25 µg/ml in 0.2 M sodium bicarbonate buffer (pH=9.5). The PS-tag²⁵ was incorporated at either the N-termini (RAFIASRRIRPGGGG-peptide) or the C-termini (peptide-GGGGRAFIASRRIRRP) with a 4xGly spacer between the PS-tag and PA binding peptide²⁶. The peptides were diluted serially across each row beginning with the 25µg/ml stock peptide solution (typically 25, 12.5, 6.25, 3.125, 1.57, 0.78, 0.39, 0.195, 0.098 µg/ml), while the antibody was diluted at a slightly lower concentration (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078, 0.039, 0.0195, 0.009, 0.0048 µg/ml). A single row of buffer was used as a negative control. Following a 2 hr incubation, each well was blocked for 1 hr using phosphate buffered saline (PBS) pH 7.4 with 0.1% Tween (PBST). For binding analysis, a PA-HRP was used at 0.2 µg/ml in PBST to determine the total PA binding to each peptide. After a 45 min incubation period, the wells were washed with PBS and detected using 1-Step Ultra TMB ELISA substrate (Pierce Protein). The data was recorded as total absorbance at 450 nm using a Synergy HT Microplate reader. The binding dissociation constant (K_D) was determined by plotting the fraction bound versus the concentration of peptide and fit using a sigmoid function with IGOR Pro.

2.1.3: PS-tag peptide specificity: PS-tag specificity was determined by diluting the PS-tag peptide serially across each row beginning with the 25µg/ml stock peptide solution (typically 25, 12.5, 6.25, 3.125, 1.57, 0.78, 0.39, 0.195, 0.098 µg/ml). For binding analysis, each of the potential anthracis protein binders, protective antigen-horseradish peroxidase (PA-HRP), lethal factor-horseradish peroxidase (LF-HRP), edema factor-horseradish peroxidase (EF-HRP), and potential cross-reacting proteins, streptavidin-horseradish peroxidase (Strep-HRP), neutravidin-horseradish peroxidase (Neutr-HRP), horseradish peroxidase (HRP), and immunoglobulin G-horseradish peroxidase (IgG-HRP) were used at 0.2 µg/ml in PBST to determine the total protein binding to the PS-tag.

2.2 PA-peptide capillary electrophoresis

2.2.1 CE method: CE experiments were completed on a P/ACE™ MDQ (Beckman Coulter, Inc; Brea, CA) equipped with a Laser-Induced Fluorescence (LIF) detector. Each CE separation was performed on a bare fused-silica capillary with 50 µm ID, 375 µm OD, L_{Tot} 37cm, and L_{eff} 20cm after an initial capillary conditioning step. The CE method included a 5 min wash with NaOH, 2 min wash with H₂O, 5 min wash with running buffer (10mM Hepes, K⁺ salt, pH 7.5), and a 2 min capillary conditioning step in running buffer at the experimental voltage prior to a 5 sec, 0.5 psi sample injection, sample separation, and data collection. Samples were analyzed using either a 10kV or 20kV separation voltage for 15 mins.

2.2.2 CE samples: Native PA (List Biological Laboratories, Inc.) protein was dye labeled with an amine reactive Dylight-488 NHS Ester (Pierce Protein) according to manufacturer's instructions. Briefly, a 1mg/ml PA sample was buffer exchanged into 0.05M sodium borate buffer at pH 8.5, added directly to a 50 µg sample of Dylight-488 NHS Ester, and reacted at room temperature for 1 hr. After the 1 hr reaction, the excess, unreacted dye was dialyzed from the PA sample using a 10,000 NMWL (nominal molecular weight limit) membrane with two buffer exchanges using 10mM Hepes, K⁺ salt, pH 7.5. The PA binding peptide was synthesized (RS Synthesis) as a native peptide and an N-terminal FITC labeled peptide at >85% purity. The peptide sample concentration was determined by dissolving the peptide in 10mM Hepes, K⁺ salt, pH 7.5 and measuring the absorbance at 280nm in a 1cm pathlength cuvette ($\epsilon = 11380 \text{ M}^{-1}\text{cm}^{-1}$ at 280nm; Peptide Property Calculator <http://www.basic.northwestern.edu/biotools/proteincalc.html>). The PA protein sample was incubated at an equal concentration of peptide (typically 3 µM each) and the peptide and a mobility shift standard, 2 µM fluorescein isothiocyanate (FITC), for 30 mins before being loaded in to the temperature controlled sample storage tray and stored at 4°C prior to each separation.

3. RESULTS AND DISCUSSION

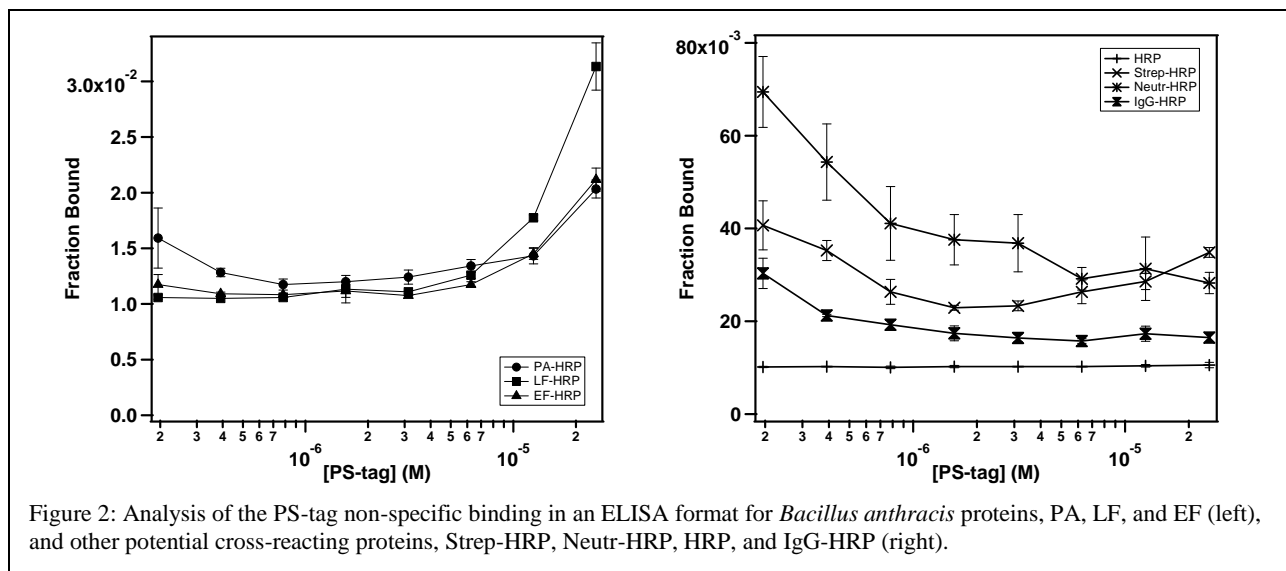
3.1 Comparison of mAb and PS-tag peptide ELISA

A side-by-side comparison using the PA monoclonal antibody, mAb, and PA binding, PS-tag peptides as an ELISA capture agent are presented in Figure 1. Both the C-terminal PS-tag sequence and N-terminal PS-tag sequence appended to the PA binding peptide resulted in nearly identical dissociation constants, $K_D = 600$ nM, as determined by fitting each curve to a sigmoid function. The peptide binding constant is approximately two-orders of magnitude less than the mAb tested, which was measured to have a $K_D = 5$ nM. One potential difference for the affinity is that each mAb contains two equivalent binding sites per antibody protein molecule, which results in greater target avidity for antibodies compared to the singularly target binding peptides. Translating the current mAb and peptide concentrations in this ELISA format, the total mass of PA detected is greater for the peptide reagent, and the PA detection limit of the peptide, by mass, is within one order of magnitude of the mAb. In this direct-ELISA format, as little as 0.1 ng of PA can be detected (per binding site) using the mAb, whereas the peptide could detect as little as 1.0 ng of PA in the 96-well plate format (Table 1).

3.2 PS-tag peptide blocks non-specific adsorption of proteins in ELISA format

Analysis of the PS-tag in control assays revealed that the PS-tag was able to bind to the ELISA plate, polystyrene surface and retain binding in the presence of all three *B. anthracis* protein toxin components, PA, LF, and EF, as well as other common assay proteins, neutr-HRP, strep-HRP, HRP, and IgG-HRP. The three *B. anthracis* proteins showed very little cross-reactivity with the PS-tag, having a maximum of 0.03 percent binding when measured by ELISA. For the streptavidin, neutravidin, and IgG, the overall background increased as the PS-tag decreased (Figure 2). Similar to the *B. anthracis* proteins, the HRP signal did not increase above the assay buffer background. Overall all of the proteins tested showed background at less than 1% of the maximum signal in the ELISA.

PA proteins showed very little PS-tag cross-reactivity or exhibited the lowest PS-tag plate displacement on the plate, along with HRP (Figure 2), all signals decreased with decreasing PS-tag. The other set of “cross-reacting proteins” analyzed showed an increased signal as the PS-tag decreased, indicating that the PS-tag is likely blocking non-specific binding to the plate. More importantly, the HRP showed no increase in background signal above the buffer background, which is critical since HRP is commonly used in ELISAs for signal amplification.



3.3 Improved Capillary Electrophoresis resolution of *B. anthracis* peptides for rapid analysis

The capillary electrophoresis mobility shift of PA-peptide complex is determined by the change in the overall mobility of the complex due to a change in the charge and/or mass of the protein complex. In Figure 3, a 0.1 min mobility shift for PA-488 is noted during a 10 kV separation after the protein-peptide complex is formed by binding to

an unlabeled PA-peptide. Alternatively, using a peptide-FITC for tracking, the resultant PA-peptide complex has a greater change in the observed mobility of the fluorescent-peptide (Figure 3). At 10kV, a 0.5 min (30 sec) mobility shift is measured between the unbound peptide-FITC and the PA-peptide-FITC complex. A more rapid analysis using 20kV results in only a 0.25 min (15 sec) mobility shift, but the increased separation voltage enables analysis of the entire PA-peptide complex in less than 3 mins with a tradeoff of resolution.

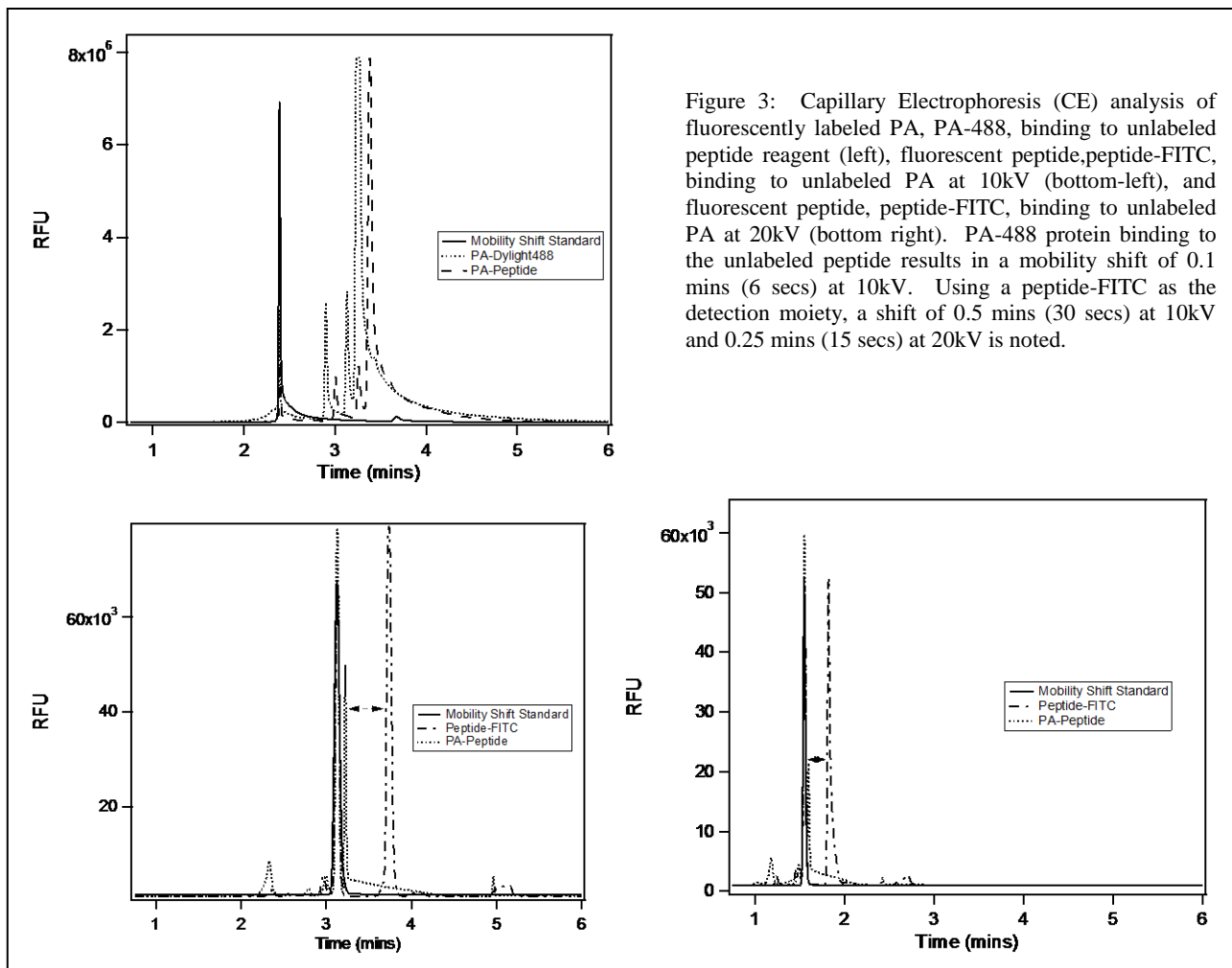


Figure 3: Capillary Electrophoresis (CE) analysis of fluorescently labeled PA, PA-488, binding to unlabeled peptide reagent (left), fluorescent peptide, peptide-FITC, binding to unlabeled PA at 10kV (bottom-left), and fluorescent peptide, peptide-FITC, binding to unlabeled PA at 20kV (bottom right). PA-488 protein binding to the unlabeled peptide results in a mobility shift of 0.1 mins (6 secs) at 10kV. Using a peptide-FITC as the detection moiety, a shift of 0.5 mins (30 secs) at 10kV and 0.25 mins (15 secs) at 20kV is noted.

4. CONCLUSIONS

Peptide recognition elements show great promise as an alternative technology to antibodies. Yet, incorporating peptides in standard antibody assays such as ELISA and affinity capillary electrophoresis (ACE) can be challenging since the small length of peptides can limit solubility in both assay formats. In ELISA, the limited number of residues can restrict the available binding sites after binding to a microwell plate surface. The PS-tag peptide fusion provides an alternative method for improving peptide reagent binding in an ELISA format and is a convenient tag that can be included in the peptide synthesis process. The PS-tag alone exhibited minimal cross-reactivity to all of the *B. anthracis* proteins tested, as well as limited binding to the protein cross-reactive panel. The increased background with decreasing PS-tag sample for the cross-reacting protein panel suggests that the PS-tag will also limit non-specific binding if is used as a blocking agent in ELISA methods.

Development of a solution-based, affinity determination complimentary method to direct peptide ELISA with PA peptides using ACE shows a lot of potential. Incorporating dye-labeled peptides as the detection reagent instead of

tracking the mobility shift of a dye-labeled protein proved to improve resolution of bound versus unbound peptide-protein complexes. Tracking the mobility change of the dye-labeled peptide resulted in greater mobility shift since the peptide mobility was dominated by the mass and charge of the protein. Solution measurements with ACE enable both equilibrium and non-equilibrium analysis of protein-peptide interactions to determine binding affinity (K_A or K_D) and binding on- and off-rates (k_{on} and k_{off}). Furthermore, binding analysis in solution rather than bound to a surface when using ELISA and SPR will provide a more accurate representation of the natural binding interaction between the target and detection molecule. ELISA and SPR remain the industry standard for binding analysis, but continued development of ACE techniques may eventually shift immunoassays from surface-bound to free-solution measurements. ACE offers a more rapid technique, within minutes for completion, with method calibration as a drawback³¹, while ELISA takes many hours and method calibration considered a strength of ELISA. Herein, we reported both peptide-detection ACE methods and peptide-capture direct ELISA methods to analyze peptide reagents selected from bacterial display libraries to recognize PA protein of *B. anthracis*.

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